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Development of tailored real-time RT-PCR assays for the detection and differentiation of serotype O, A and Asia-1 foot-and-mouth disease virus lineages circulating in the Middle East

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ABSTRACT

Rapid and accurate diagnosis is essential for effective control of foot-and-mouth disease (FMD). In countries where FMD is endemic, identification of the serotypes of the causative virus strains is important for vaccine selection and tracing the source of outbreaks. In this study, real-time reverse transcription polymerase chain reaction (rRT-PCR) assays using primer/probe sets designed from the VP1 coding region of the virus genomes were developed for the specific detection of serotype O, A and Asia-1 FMD viruses (FMDVs) circulating in the Middle East. These assays were evaluated using representative field samples of serotype O strains belonging exclusively to the PanAsia-2 lineage, serotype A strains of the Iran-05 lineage and serotype Asia-1 viruses from three relevant sub-groups. When RNA extracted from archival and contemporary field strains was tested using one- or two-step rRT-PCR assays, all three primer/probe sets detected the RNA from homotypic viruses and no cross-reactivity was observed with heterotypic viruses. Similar results were obtained using both single- and multiplex assay formats. Using plasmid standards, the minimum detection level of these tests was found to be lower than two copies. The results illustrate the potential of tailored rRT-PCR tools for the detection and categorization of viruses circulating in the Middle East belonging to distinct subgroups of serotypes O, A and Asia-1. These assays can also overcome the problem of serotyping samples which are found positive by the generic rRT-PCR diagnostic assays but negative by virus isolation and antigen-detection ELISA which would otherwise have to be serotyped by nucleotide sequencing. A similar approach could be used to develop serotyping assays for FMDV strains circulating in other regions of the world.

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1. Introduction

Foot-and-mouth disease (FMD) is a highly contagious vesicular disease of wild and domestic cloven-hoofed animals, particularly cattle, sheep, pigs and goats. It is the most economically important viral disease of domesticated livestock throughout the world today, being endemic in many countries of Africa, Asia and South

America. However, outbreaks can also occur in countries that are normally free of FMD: including recently Japan and Korea (2000 and 2010), the United Kingdom (UK: 2001 and 2007), France and The Netherlands (2001) plus Bulgaria (2010–2011). The causative agent, FMD virus (FMDV), is a single-stranded positive-sense RNA virus of around 8.4 kilobases in length, belonging to the genus *Aphthovirus* within the family *Picornaviridae* (Belsham, 2005). There are seven immunologically distinct serotypes: O, A, C, SAT (Southern African Territories) 1, SAT 2, SAT 3 and Asia-1 which encompass a diverse antigenic spectrum of virus strains. The seven serotypes are not distributed equally around the world (Knowles and Samuel, 2003). Serotypes O and A are widely disseminated but type C viruses have not been detected since 2004, while Asia-1, despite having been detected in Greece, is mainly confined to the Asian continent.

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The SAT serotypes are normally, but not exclusively, restricted to sub-Saharan Africa.

In countries normally free of FMD, rapid confirmation of FMDV as the causative agent in material from suspect disease cases is the primary goal of field and laboratory investigations. A number of generic (pan-serotypic) real-time reverse transcription polymerase chain reaction (rRT-PCR) assays have been developed to target highly conserved regions of the RNA genome of FMDV (Reid et al., 2002; Callahan et al., 2002; Moniwa et al., 2007) which efficiently detect all seven serotypes in clinical samples. These assays have undergone extensive evaluation; parallel testing of samples has shown that the sensitivity of these molecular assays is at least equal to that of the current “gold standard” method of virus isolation in cell culture (Shaw et al., 2004; Ferris et al., 2006; King et al., 2006; Reid et al., 2009). However, a limitation of the rRT-PCR procedures targeting conserved sequences in untranslated regions of the genome or those encoding non-structural proteins is that they cannot determine the serotype of the causative FMDV. Therefore, using these assays it is not possible to identify the serotype of virus samples that are positive by rRT-PCR but virus isolation (VI)/antigen-detection ELISA (Ag-ELISA) negative. Serotyping of these samples may only be accomplished by the use of serotype-specific primers and probes and/or nucleotide sequencing. Serotype-specific primer/probe sets may also be specifically beneficial to those countries that currently use only the generic rRT-PCR assays or a combination of generic rRT-PCR and Ag-ELISA but not VI.

In FMD-endemic countries, identification of the serotypes of the causative virus strains is important for vaccine selection, disease containment and for tracing the source of the outbreaks. Conventional RT-PCR procedures using primers corresponding to the VP1 (1D) coding region and to the 2A/2B coding regions for serotyping of FMDV have been reported (Vangrype and De Clercq, 1996; Callens and De Clercq, 1997). However, subsequent studies have shown that these agarose gel-based assays have relatively poor sensitivity and specificity due to the genetic diversity within all FMDV serotypes (Reid et al., 1999, 2001). While these procedures can be used in conjunction with Ag-ELISA and VI to provide additional information, they are insufficiently sensitive to replace them for primary diagnosis of FMD (Reid et al., 1999). Alternative RT-PCR assays reported by Suryanarayana et al. (1999) and Alexandersen et al. (2000) for serotype-specific diagnosis were cumbersome and unsuitable for routine use in the event of an epidemic. A conventional RT-PCR procedure for differentiation of FMDV serotypes native to India using multiple primers based mostly on nucleotide sequences of viruses circulating in that geographical area was described by Giridharan et al. (2005). These studies demonstrated the potential for using tailored molecular tools to identify specific serotypes as an alternative or addition to pan-serotypic assays for detection of FMDV. More recently, the development and evaluation of a real-time reverse transcription-loop-mediated isothermal amplification assay for rapid serotyping of FMDV has been reported (Madhanmohan et al., 2013).

The objective of this study was to develop tailored rRT-PCR methods for identification of FMDV serotypes. In addition to using these tools in endemic countries, such an approach would overcome the problem of serotyping samples which are positive by pan-serotypic rRT-PCR but cannot be sequenced using standard protocols. This collaborative study between the WRLFMD (The Pirbright Institute, UK), Technical University of Denmark (DTU) and Khorasan Razavi Central Veterinary Laboratory in Iran (CVL) describes the development and evaluation of tailored serotype-specific rRT-PCR assays for the detection of archival and contemporary strains of FMDV serotypes O, A and Asia-1 circulating in the Middle East.

2. Materials and methods

2.1. Virus isolates and sample preparation

FMDV isolates were selected from archival stocks held in the WRLFMD (Pirbright Institute, UK) repository at -20°C and contemporary submissions from within the serotype O PanAsia-2 and serotype A Iran-05 lineages, respectively, serotype Asia-1 subgroups 1, 2 and 6, plus representative FMDV-Genome Detected samples of undetermined serotype (FMDV-GD: Ag-ELISA and VI negative). To check the diagnostic specificity of the serotype-specific primer/probe sets, archival and currently circulating serotype O and A viruses belonging to other topotypes (geographically and genetically distinct evolutionary lineages; Knowles and Samuel, 2003) and serotype Asia-1 viruses from other subgroups were also tested. A further check on the specificity of the primer/probe sets was made by testing FMDV strains belonging to serotypes C, SAT 1 and SAT 2 plus representative strains of swine vesicular disease virus (SVDV) which are capable of causing clinically indistinguishable (‘look-alike’) disease to that caused by FMDV in pigs and some samples, designated ‘NVD’, from which no virus was detected by either Ag-ELISA, VI or pan-serotypic rRT-PCR. Most samples comprised $\sim 10\%$ (w/v) original suspensions of vesicular epithelium (OS; Ferris and Dawson, 1988) which had been tested by Ag-ELISA and VI (Ferris and Dawson, 1988) at the time of receipt. Additional clinical samples such as saliva samples or homogenates generated from heart tissue were also tested, and cell culture supernatants of selected strains were tested when OS was unavailable. All the samples tested are listed in the supplementary data—Annexes A and B.

2.2. Total nucleic acid extraction

Total nucleic acid was extracted from the samples by an automated procedure (Shaw et al., 2007). Prior to nucleic acid extraction, 0.2 ml of OS, saliva or cell culture-grown viruses were added per 0.3 ml of Lysis/Binding Buffer (Roche) or 0.2 ml of OS or cell culture-grown virus was added to 1.0 ml of TRIzol[®] Reagent (Invitrogen) and stored at -70°C . Nucleic acid extraction was also performed manually using a QIAamp Viral RNA MiniKit (Qiagen) according to the manufacturer’s instructions.

2.3. Primer and probe design

GenBank sequences (<http://www.ncbi.nlm.nih.gov/>) were aligned from the VP1 coding region of the FMDV genome for serotype O PanAsia-2, serotype A Iran-05 and Asia-1 strains of subgroups 1, 2 and 6, respectively, using BioEdit (<http://www.mbio.ncsu.edu/bioedit.html>). Hydrolysis (TaqMan[®]) probes and primers were designed from conserved sequences using PrimerExpress version 3.0 software (Applied Biosystems).

In pilot experiments, for serotypes O and A, two candidate forward primers, two reverse primers and one probe were designed for evaluation. Four forward primers, two reverse primers and four probes were evaluated in all combinations for detection of Asia-1 virus sequences of the three targeted subgroups of this serotype. The best-performing primer/probe sets for each serotype were selected after all sets were tested by one-step rRT-PCR using an amplification protocol adapted for routine diagnosis of FMDV (Shaw et al., 2007) with template nucleic acid extracted from serotype O PanAsia-2, serotype A Iran-05 and Asia-1 viruses (subgroups 1, 2 and 6, including the recently circulating Asia-1/Sindh-08 lineage), respectively (data not shown). Table 1 lists the sequences of the best-performing primer/probe sets.

Table 1
FMDV serotype-specificity and sequence of the primers/probe sets.

Primer/probe name	Orientation	FMDV serotype specificity	Sequence (5'–3')
TypeOforprimer1	Forward	O ^a	CCGAGACAGCGTTGGATAACA
TypeOrevprimer1	Reverse	O	CCATACTTGACGTTCCCGTTGT
PanAsiaOprob349	Forward	O	FAM-CCGACTTGCACTGCCTTACACGGC-TAMRA
TypeAforprimer2	Forward	A ^b	ACGACCATCCACGAGCTYC
TypeArevprimer2	Reverse	A	RCAGAGGCTGGGACAGTAG
Alran05probe	Forward	A	FAM-CGTGCGCATGAAACGTGCCG-TAMRA
Asia1forward3	Forward	Asia-1 ^c	GCACTWAAGGCGYAGASCATYAC
Asia1reverse2	Reverse	Asia-1	GCARAGGCTAGGGCAGTATG
Asia1probe4	Forward	Asia-1	FAM-AGCTGTTGATCCGCATGAAACGYGCG-TAMRA

^a FMDV serotype O PanAsia-2 lineage.

^b FMDV serotype A Iran-05 lineage.

^c FMDV serotype Asia-1 subgroups 1, 2 and 6.

2.4. Evaluation of the FMDV serotype-specific primer/probe sets in assays on clinical samples

Clinical samples were tested at the WRLFMD (Pirbright Institute, UK) by one-step rRT-PCR assays using the serotype-specific primer/probe sets run in parallel with the generic FMDV primer/probe sets targeting the 5' UTR (Reid et al., 2002) and 3D coding region (Callahan et al., 2002) using the protocol of Shaw et al. (2007). The pan-serotypic assays, targeting independently conserved regions of the FMDV genome, also served to determine the presence of FMDV RNA. All rRT-PCR reactions were set-up manually and amplifications performed in a Stratagene Mx3005P thermal cycler.

At DTU (Lindholm, Denmark), two-step rRT-PCR assays were performed with random primers for cDNA preparation followed by rRT-PCR amplification targeting the 5' UTR (Reid et al., 2002), 3D coding region (Callahan et al., 2002) or using the serotype-specific primer/probe sets. Fifty cycles of replication were used in a Stratagene Mx3005P thermal cycler.

The CVL (Iran) methodology used both one- and two-step rRT-PCR protocols with the best-performing serotype O, A and Asia primers/probe sets from Table 1. Amplification by rRT-PCR was performed in a Lightcycler 480 (Roche) or ABI 7500 (Applied Biosystems) thermal cyclers with the following amplification programme: an RT step of 50 °C for 30 min; denaturation at 95 °C for 15 min, 1 cycle; 94 °C for 30 s, 60 °C for 1 min, 50 cycles.

2.5. Evaluation of FMDV serotype-specific primer/probe sets in multiplex assays

The individual serotype-specific assays were combined together in a multiplex format that allowed single RNA samples to be simultaneously tested for all three targets within the same tube. The reactions were set up as a multiplex assay with the probe targeting FMDV serotype O labelled with 5'-Cy5 and 3'-Black Hole Quencher (BHQ) 2, the probe targeting FMDV serotype A labelled with 5'-FAM and 3'-BHQ1 and the probe targeting FMDV serotype Asia-1 labelled with 5'-Cy3 and 3'-BHQ2 (Sigma-Aldrich, UK). Cycling conditions used in these assays were identical to those used for the individual assays. The diagnostic performance of the multiplex

assay was compared to the individual component (singleplex) rRT-PCR assays.

2.6. Preparation of RT-PCR products suitable as standards for the serotype-specific rRT-PCR assays

RT reactions (in 20 µl) were set up containing 4 µl of RNA from O/IRN/8/2005, A/IRN/1/2005 and Asia 1/IRN/10/2004 using SuperScript™ III reagents (Invitrogen, Paisley, UK) according to the manufacturer's instructions with 10 µM each of the reverse primers (Table 2). Primers, RNA, water and dNTPs were first combined and the mixture heated to 65 °C for 5 min, followed by chilling on ice. The remaining reagents were subsequently added and heated at 50 °C for 60 min followed by a 15 min inactivation step at 95 °C. PCRs were prepared using a Platinum® Taq system (Invitrogen, Paisley, UK) according to the manufacturer's instructions with 10 µM each of the primers (Table 2). Amplification resulted in the production of fragments of 231, 168 and 197 bp for serotypes O, A and Asia-1, respectively, corresponding to the assay target region. The PCR cycling protocol (Bio-Rad Laboratories, Herts, UK) included 30 cycles consisting of 30 s each of denaturation at 94 °C, annealing at 55 °C and extension at 68 °C. On completion of cycling, an additional extension step of 10 min at 68 °C was carried out followed by a final hold step at 4 °C. Resultant PCR products were visualised on 2% (w/v) agarose gels (Sigma-Aldrich, UK), before being excised from the agarose gel and purified using a Qiaquick Gel Extraction Kit (Qiagen, Crawley, UK) according to the manufacturer's instructions.

2.7. Preparation of plasmid standards for determination of analytical sensitivity of serotype-specific rRT-PCR assays

The amplified assay target regions were cloned into a pGEMT Easy vector (Promega, UK) according to the manufacturer's instructions. A number of positive colonies were grown overnight in 3 ml of LB-broth containing 50 µg/ml of ampicillin. Plasmids were purified using a Qiagen plasmid mini kit (Qiagen, Crawley, UK) according to the manufacturer's instructions and tested for the presence of the assay target region by PCR with the same primers. The correct sequence and orientation was confirmed by sequencing with M13 forward and reverse primers (Promega, UK) on a 3730 DNA analyser (Applied Biosystems, Warrington, UK) by dideoxy-sequencing

Table 2
FMDV serotype specificity, sequence and amplicon size with the primer sets used to make standards.

Primer name	Sequence (5'–3')	FMDV serotype specificity	Size (bp)
SeroOquantfor	TCG CAG ACC TCG AGG TGG	O (IRN 8/2005)	231
SeroOquantrev	CTT GCA GGT CAC CTC TCA CG	O (IRN 8/2005)	
PEXSeroAquantfor	GGG GTG ACC TGG GGC CTC TTG CG	A (IRN 1/2005)	168
PEXSeroAquantrev	TGT CCT GCG ACA ACA CTT CCA C	A (IRN 1/2005)	
PEXSeroAsia1quantfor	GGA AAC AAC CTC ACG GCG TGG TG	Asia 1 (IRN 10/2004)	197
PEXSeroAsia1quantrev	TCT CCT GTT TAC GGC GAT CCT G	Asia 1 (IRN 10/2004)	

using a Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) following the manufacturer's instructions.

RNA was transcribed from 0.4 µg of linearised pGEMT plasmids from the T7 promoter site using a MEGAscript® T7 *in vitro* transcription kit (Ambion, Warrington, UK) according to the manufacturer's instructions except that transcription was continued overnight. Transcribed RNA was quantified using a Nanodrop (Thermo Scientific, UK) and verified by running 1 µl RNA on a BioAnalyser (Agilent, UK) using the RNA 6000 Nano kit (Agilent, UK).

2.8. Determination of the dynamic range and analytical sensitivity of the serotype-specific assays using the RNA and plasmid standards

Dilutions of the RNA transcripts containing between 10^{12} and 10^0 copies/µl were used as target material to test the dynamic range and sensitivity of the single- and multiplex assays. Real-time RT-PCR was carried out using Superscript III Platinum® One-Step qRT-PCR reaction mix and 0.5 µl enzyme (Invitrogen, Paisley, UK). The final reaction volume (25 µl) included 1.5 µl of probe at 5 pmol/µl; 2 µl of each primer at 10 pmol/µl, 12.5 µl 2× reaction mix, 1.5 µl of DEPC-water (Ambion, Warrington, UK) per reaction and 5 µl of target RNA. Real-time RT-PCR reactions, including standard RNA material of between 10^{12} and 10^0 copies/µl, were carried out in quadruplicate in an Mx3005p real-time thermal cycler (Stratagene, UK). For these experiments, the thermal cycling conditions included a single 60 °C cDNA synthesis step for 30 min followed by a 95 °C denaturation step of 10 min before PCR cycling which included 50 cycles consisting of 15 s each of denaturation at 95 °C, and annealing/extension at 60 °C for 1 min (as previously described; Shaw et al., 2007).

3. Results

3.1. Diagnostic sensitivity and specificity of serotype-specific rRT-PCR primer/probe assays in singleplex and multiplex assay formats

3.1.1. Singleplex rRT-PCR assay format

RNA samples derived from known or suspect cases of FMD were assayed initially in singleplex rRT-PCR assays. Threshold cycle (C_T) values were assigned to each reaction as previously described (Reid et al., 2002). As for routine diagnosis using the pan-serotypic one-step rRT-PCR assays, a C_T value of 32.0 was used as the positive/negative cut-off point (Shaw et al., 2007; Reid et al., 2009). A definitive positive/negative cut-off was not assigned to the serotype-specific rRT-PCR assays; the strongest result (i.e. lowest C_T value) generated for each sample from either the serotype O, A or Asia-1-specific primer/probe set was used to designate an O, A or Asia-1 serotype for that sample.

Singleplex assay data generated from WRLFMD, DTU and CVL are summarised in Table 2 and the results from the individual samples are listed in supplementary data—Annex A. Assays with the pan-serotypic primers and probes confirmed the presence of FMDV RNA within each sample. As expected, both pan-serotypic assays (targeting the 5' UTR and 3D coding regions) demonstrated a broader spectrum of reactivity than the serotype-specific primer/probe sets and detected the genomes of serotype C, SAT 1 and SAT 2 viruses, as well as being broadly reactive against serotype O, A and Asia-1 viruses across all topotypes/lineages. All three serotype-specific primer/probe sets tested were strongly positive using RNA extracted from homotypic serotype O, A and Asia-1 viruses of the targeted lineage or subgroup. There were only three instances, all involving the serotype O-specific primers/probe set,

when homotypic viruses of the non-targeted lineage, were detected (viruses belonging to the topotypes EA-3, EA-4 and CATHAY).

The specific primer/probe sets were successful in identifying four of the 12 FMDV-GD samples tested as serotype O and seven of these samples were serotype A. Of the 12 FMDV-GD samples tested by the singleplex format, 9 and 10 samples, respectively, were positive by the 5' UTR and 3D pan-serotypic PCR assays, however, two samples testing positive using the serotype A-specific primer/probe set were negative in assays with pan-serotypic primer/probe sets.

No cross-reactivity was observed when the specific primer/probe sets were tested with heterotypic type O, A or Asia-1 isolates except when testing the serotype O PanAsia-2 sample O/PAK/4/2006 which also produced a weak reaction (of higher C_T value) against the serotype A-specific primers/probe set. This sample may have been a dual serotype O/A infection but as a stronger C_T value (28.33) was produced with the serotype O primer/probe set than that resulting from the heterologous serotype A set (C_T value of 31.55), a serotype O identification could be correctly deduced for this sample. Furthermore, no cross-reactivity was observed when all three serotype-specific primer/probe sets were tested against the heterologous FMDV serotypes C, SAT 1 and SAT 2 or with SVDV isolates (Table 3).

A two-step singleplex rRT-PCR format was also used at DTU and at CVL in parallel with the one-step format (data summarised in Table 3). Like the one-step approach, the two-step assays with the specific primer/probe sets were strongly reactive with the targeted type O strains of PanAsia-2 lineage, type A strains of the Iran-05 lineage and Asia-1 viruses from subgroups 1 and 2, respectively, but overall assay performance was better with the one-step format (data not shown). No cross-reactivity was observed when the serotype-specific primer/probe sets were tested using the two-step format assays against the heterologous serotype C, SAT 1, SAT 2 and SAT 3 FMDVs.

3.1.2. Multiplex rRT-PCR assay format

The positive/negative acceptance criteria adopted for the pan-serotypic and serotype-specific rRT-PCR assays using the singleplex format were similarly applied for the multiplex assays performed at the WRLFMD. Multiplex assay data are summarised in Table 3 and the C_T values from individual samples are listed in supplementary data—Annex B. Specificity of detection using the serotype O, A and Asia-1 primer/probe sets is also apparent from the heat maps (Fig. 1a–c, respectively). These clearly demonstrate the strong specificity of recognition of the primer/probe sets against the targeted virus sequences of the isolates belonging to the serotype O PanAsia-2 lineage (Fig. 1a), serotype A Iran-05 lineage (Fig. 1b) and Asia-1 subgroups 1 and 2 (Fig. 1c), respectively. However, one sample (O/BAR/1/2008) did not generate a positive result for the multiplex assay although the singleplex version of this test gave a positive signal.

The samples Asia-1/PAK/32/2011 and Asia-1/PAK/109/2011 were among five Asia-1 viruses in the Asia-1/Sindh-08 lineage not detected by the Asia-1-specific primers/probe sets. Both viruses had two mismatches in the probe-binding region resulting in false-negative rRT-PCR results. Table 4 shows the sequences of selected Asia-1 viruses, highlighting mismatches in the primer and probe regions. All other Asia-1 viruses listed in Table 4 were detected by the multiplex assay.

The multiplex assay format was also successful in assigning the serotypic identity to samples designated as FMDV-GD (Table 3, supplementary data—Annex B and heat map; Fig. 1d). Eighty-five FMDV-GD samples were tested (77 and 82 had tested positive by the 5' UTR and 3D assays, respectively) and of these, serotype O identity was assigned to 33 samples, 17 were designated as serotype A and four samples were designated as serotype Asia-1.

Table 3
Summary of the rRT-PCR results using serotype O-, A- and Asia-1-specific and pan-serotypic 5' UTR and 3D primers/probe sets in multiplex and singleplex assay formats.

FMDV serotype or other vesicular virus	FMDV toptotype ^a	FMDV lineage ^b	Ratio of number of samples positive to total number tested by rRT-PCR ^c					Ratio of number of samples positive to total number tested by rRT-PCR ^c				
			Multiplex assay format ^d					Singleplex assay format ^{e,f}				
			O ^g	A ^h	Asia-1 ⁱ	5'UTR	3D	O ^g	A ^h	Asia-1 ⁱ	5'UTR	3D
O	ME-SA	PanAsia-2	12/13	0/13	0/13	13/13	13/13	26/26 ^e 5/5 ^f	1/26 ^e 0/5 ^f	0/26 ^e 0.5 ^f	23/26 ^e 5/5 ^f	24/26 ^e 5/5 ^f
	ME-SA	PanAsia-2 ^{ANT-10}	19/19	0/19	0/19	18/19	19/19	17/18 ^e	0/18 ^f	0/18 ^f	NT ^j	18/18 ^f
	ME-SA	PanAsia-2 ^{FAR-09}	2/2	0/2	0/2	2/2	2/2	NT	NT	NT	NT	NT
	ME-SA	PanAsia	5/5	0/5	0/5	5/5	5/5	NT	NT	NT	NT	NT
	ME-SA	Unnamed	1/1	0/1	0/1	1/1	1/1	NT	NT	NT	NT	NT
	ME-SA	Ind-2001d	1/1	0/1	0/1	1/1	1/1	NT	NT	NT	NT	NT
	SEA	Unnamed	0/2	0/2	0/2	2/2	2/2	0/1 ^e	0/1 ^e	0/1 ^e	1/1 ^e	1/1 ^e
	SEA	Mya-98	1/10	0/10	0/10	10/10	10/10	NT	NT	NT	NT	NT
	EA-1	Unnamed	0/1	0/1	0/1	1/1	1/1	0/1 ^e	0/1 ^e	0/1 ^e	1/1 ^e	1/1 ^e
	EA-2	Unnamed	0/2	0/2	0/2	2/2	2/2	0/2 ^e	0/2 ^e	0/2 ^e	2/2 ^e	2/2 ^e
	EA-3	Unnamed	0/1	0/1	0/1	1/1	1/1	1/2^e	0/2 ^e	0/2 ^e	2/2 ^e	2/2 ^e
	EA-4	Unnamed	0/1	0/1	0/1	1/1	1/1	1/1^e	0/1 ^e	0/1 ^e	1/1 ^e	1/1 ^e
	WA	Unnamed	0/1	0/1	0/1	1/1	1/1	0/1 ^e	0/1 ^e	0/1 ^e	1/1 ^e	1/1 ^e
	ISA-1	Unnamed	0/1	0/1	0/1	1/1	1/1	0/1 ^e	0/1 ^e	0/1 ^e	1/1 ^e	1/1 ^e
	ISA-2	Unnamed	0/1	0/1	0/1	1/1	1/1	0/1 ^e	0/1 ^e	0/1 ^e	1/1 ^e	1/1 ^e
	CATHAY	Unnamed	0/3	0/3	0/3	3/3	3/3	1/2^e	0/2 ^e	0/2 ^e	2/2 ^e	2/2 ^e
A	ASIA	Iran-05 ^{AFG-07}	0/16	16/16	0/16	15/16	16/16	NT	NT	NT	NT	NT
	ASIA	Iran-05 ^{FAR-09}	0/1	1/1	0/1	1/1	1/1	NT	NT	NT	NT	NT
	ASIA	Iran-05 ^{BAR-08}	0/4	4/4	0/4	4/4	4/4	NT	NT	NT	NT	NT
	ASIA	Iran-05	0/17	17/17	0/17	17/17	17/17	0/22 ^e 0/9 ^f	22/22 ^e 8/9 ^f	0/22 ^e 0/9 ^f	22/22 ^e 5/9 ^f	22/22 ^e 8/9 ^f
	ASIA	Iran-05 ^{EZM-07}	0/1	1/1	0/1	1/1	1/1	NT	NT	NT	NT	NT
	ASIA	Iran-05 ^{QAZ-11}	0/2	2/2	0/2	2/2	2/2	NT	NT	NT	NT	NT
	ASIA	Iran-05 ^{ESF-10}	0/1	1/1	0/1	1/1	1/1	NT	NT	NT	NT	NT
	ASIA	Iran-87	0/1	0/1	0/1	1/1	1/1	0/1 ^e	0/1 ^e	0/1 ^e	1/1 ^e	1/1 ^e
	ASIA	Iran-96	NT	NT	NT	NT	NT	0/3 ^e 0/2 ^f	0/3 ^e 0/2 ^f	0/3 ^e 0/2 ^f	2/3 ^e 2/2 ^f	3/3 ^e 2/2 ^f
	ASIA	Sea-97	0/4	2/4	0/4	4/4	4/4	NT	NT	NT	NT	NT
	AFRICA	G-VIII	0/2	1/2	0/2	2/2	2/2	0/2 ^e	1/2^e	0/2 ^e	2/2 ^e	2/2 ^e
	EURO-SA	A ₂₄	0/1	1/1	0/1	1/1	1/1	0/1 ^e	1/1^e	0/1 ^e	1/1 ^e	1/1 ^e
	EURO-SA	A ₁₂	0/1	1/1	0/1	1/1	1/1	0/1 ^e	1/1^e	0/1 ^e	1/1 ^e	1/1 ^e
	AFRICA	G-VII	0/2	1/2	0/2	2/2	2/2	0/2 ^e	1/2^e	0/2 ^e	2/2 ^e	2/2 ^e
	AFRICA	G-II	0/1	0/1	0/1	1/1	1/1	1/1^e	0/1 ^e	0/1 ^e	1/1 ^e	1/1 ^e
	AFRICA	G-VI	0/2	0/2	0/2	2/2	2/2	0/2 ^e	0/2 ^e	0/2 ^e	2/2 ^e	2/2 ^e
	AFRICA	G-V	0/1	1/1	0/1	1/1	1/1	0/1 ^e	1/1^e	0/1 ^e	1/1 ^e	1/1 ^e
	AFRICA	G-IV	0/1	0/1	0/1	1/1	1/1	NT	NT	NT	NT	NT
Asia-1	ASIA	Unnamed	0/4	0/4	4/4	4/4	4/4	0/1 ^e	0/1 ^e	0/1 ^e	1/1 ^e	1/1 ^e
	ASIA	Sindh-08	0/12	0/12	7/12	12/12	12/12	NT	NT	NT	NT	NT
	ASIA	Group 1	NT	NT	NT	NT	NT	0/4 ^e 0/1 ^f	0/4 ^e 0/1 ^f	4/4 ^e 1/1 ^f	4/4 ^e 1/1 ^f	4/4 ^e 1/1 ^f
	ASIA	Group 2	0/2	0/2	2/2	2/2	2/2	0/6 ^e 0/6 ^f	0/6 ^e 0/6 ^f	6/6 ^e 6/6 ^f	6/6 ^e 1/2 ^f	6/6 ^e 6/6 ^f
	ASIA	Group 4	NT	NT	NT	NT	NT	0/1 ^e	0/1 ^e	1/1 ^e	1/1 ^e	1/1 ^e
	ASIA	Group 5	NT	NT	NT	NT	NT	0/1 ^e	0/1 ^e	1/1 ^e	1/1 ^e	1/1 ^e
	ASIA	Group 6	NT	NT	NT	NT	NT	0/2 ^e	0/2 ^e	2/2 ^e	2/2 ^e	2/2 ^e
C			NT	NT	NT	NT	NT	0/1 ^e 0/1 ^f	0/1 ^e 0/1 ^f	0/1 ^e 0/1 ^f	1/1 ^e 1/1 ^f	1/1 ^e 1/1 ^f
SAT 1			0/3	0/3	0/3	2/3	3/3	0/4 ^e 0/1 ^f	0/4 ^e 0/1 ^f	0/4 ^e 0/1 ^f	2/4 ^e 1/1 ^f	4/4 ^e 1/1 ^f
SAT 2			0/3	0/3	0/3	3/3	3/3	0/4 ^e 0/4 ^f	0/4 ^e 0/4 ^f	0/4 ^e 0/4 ^f	4/4 ^e 1/1 ^f	4/4 ^e 4/4 ^f
SAT 3			NT	NT	NT	NT	NT	0/1 ^f	0/1 ^f	0/1 ^f	1/1 ^f	1/1 ^f
FMDV-GD ^k			33/85	17/85	4/85	77/85 ^k	82/85 ^k	4/12 ^e	7/12 ^{e,m}	0/12 ^e	9/12 ^{e,m}	10/12 ^{e,m}
SVDV ⁿ			0/3	0/3	0/3	0/3	0/3	0/3 ^e	0/3 ^e	0/3 ^e	0/3 ^e	0/3 ^e
NVD ^o			0/4	0/4	0/4	0/4	0/4	NT	NT	NT	NT	NT

^a FMDV toptypes: ME-SA, Middle East-South Asia; SEA, South East Asia; EA-1, EA-2, EA-3 and EA-4, East Africa 1–4; WA, West Africa; ISA1, ISA-2, Indonesia 1 and 2.

^b http://www.wrlfmd.org/fmd_genotyping/prototypes.htm.

^c For pan-serotypic rRT-PCR assays, samples producing a C_T value <32.0 were considered positive; with all serotype-specific rRT-PCR assays, the lowest C_T value generated amongst the primers/probe sets used defined the serotype. Results in bold type represent apparent anomalous results where positive results were achieved by the specific primer/probe set using RNA extracted from homotypic serotype O, A and Asia-1 viruses homotypic viruses of the non-targeted lineage or subgroup.

^d All multiplex data were generated at the WRLFMD.

^e Singleplex data generated at the WRLFMD using one-step rRT-PCR assays.

^f Singleplex data generated from DTU and CVL using either one- or two-step rRT-PCR assays.

^g Primers/probe set: TypeOforprimer1/TypeOrevprimer1/PanAsiaOprob349.

^h Primers/probe set: TypeAforprimer2/TypeArevprimer2/Alran05probe.

ⁱ Primers/probe set: Asia1forward3/Asia1reverse2/Asia1probe4.

^j NT, not tested.

^k FMDV-GD, FMDV-genome detected samples, i.e. samples of undetermined serotype that are positive by rRT-PCR but VI/Ag-ELISA-negative.

^l Thirty-six samples were FMDV-genome detected (by at least one generic primers/probe set) but negative using serotype-specific primers/probe sets.

^m Two samples were positive using TypeAforprimer2/TypeArevprimer2/Alran05probe set were negative using pan-serotypic primer/probe sets.

ⁿ SVDV, swine vesicular disease virus.

^o NVD, no virus detected in the sample.

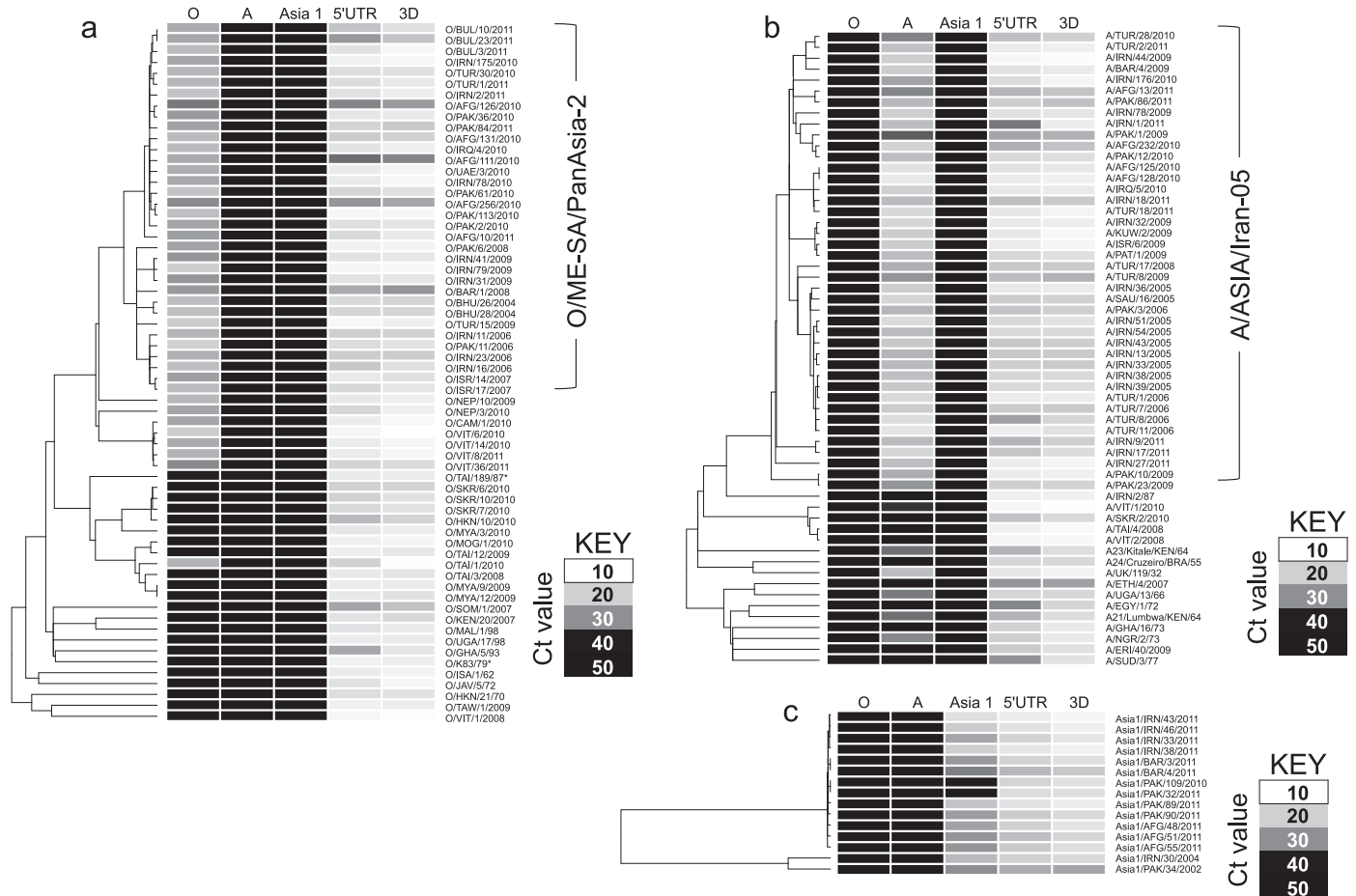


Fig. 1. Diagnostic sensitivity and specificity of the lineage-specific rRT-PCR assays in multiplexed format (together with comparative data for the pan-serotypic rRT-PCR assays) to detect representative FMD virus positive samples from serotype O PanAsia-2 lineage (a), serotype A Iran-05 lineage (b) and Asia-1 subgroups 1 and 2 (c). Data are presented as a heat-map for C_t values generated with the different rRT-PCR assays (scale shown). Neighbor-joining trees shown represent genetic relationships between VP1 sequences for the individual viruses. Individual data values are presented in supplemental data.

Table 4

Sequences of serotype Asia-1 FMDV samples highlighting mismatches with primers and probe sequences. Samples Asia-1/PAK/32/2011 and Asia-1/PAK/109/2011 were not detected by the multiplex rRT-PCR assay.

Asia-1 FMDV sample	Forward primer (5' -3')	Probe (5' – 3')	Reverse primer 3' -5')
	GCAGTWAAGGCGAGASCATYAC	AGCTGTTGATCCGCATGAAACGYGCG	CATACTGCCCTAGGCCTYTCG
Asia-1/PAK/89/2011A.A.....C.....C.....
Asia-1/AFG/48/2011A.A.....C.....C.....
Asia-1/AFG/51/2011A.A.....C.....C.....
Asia-1/AFG/55/2011A.A.....C.....C.....
Asia-1/BAR/3/2011A.A.....	..T..C.....T..C.....
Asia-1/BAR/4/2011A.A.....	..T..C.....T..C.....
Asia-1/IRN/33/2011A.A.....	..T..C.....C.....
Asia-1/IRN/38/2011A.A.....	..T..C.....C.....
Asia-1/IRN/43/2011A.A.....	..T..C.....C.....
Asia-1/IRN/46/2011A.A.....	..T..C.....C.....
Asia-1/PAK/90/2011A.A.....	..T..C.....C.....
Asia-1/PAK/32/2011A.A.....C.....A.....C.....
Asia-1/PAK/109/2010A.A.....C.....A.....C.....

3.2. Analytical sensitivity/minimum detection level of serotype-specific rRT-PCR primers/probe assays

Fig. 2a–c shows the wide dynamic range of the singleplex assays using plasmid standards with the serotype O-, A- and Asia-1-specific primer/probe sets. Using DNA templates,

the three assays had a limit of detection of 1.78, 2.58 and 2.74 copies, respectively, thus demonstrating the high analytical sensitivity of these tests. The amplification efficiencies for these three assays were estimated to be 94.9, 93.3 and 99.2%, for the serotype O, A and Asia-1 assays, respectively. Of the two formats, the singleplex assay format had a lower

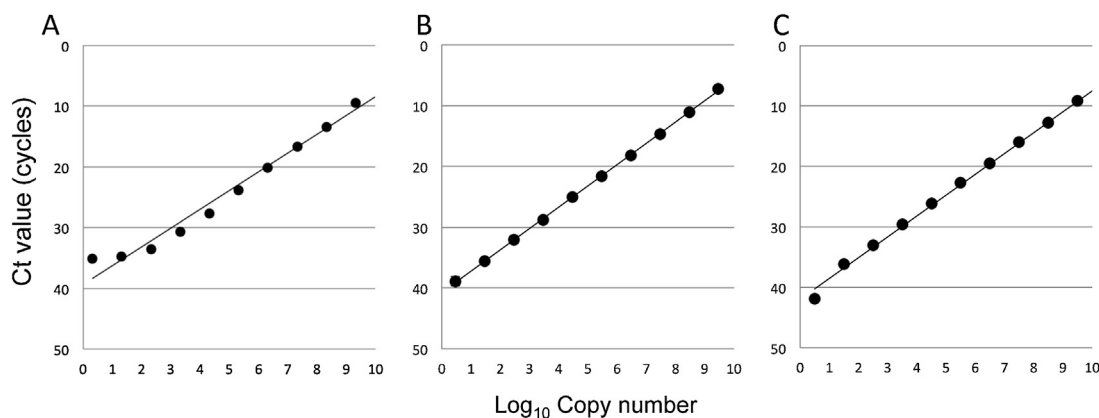


Fig. 2. Analytical sensitivity and demonstration of the dynamic range of the rRT-PCR assays using plasmid standards with the serotype O- (a), A- (b) and Asia-1- (c) specific primer/probe sets.

minimum detection level than the multiplex version (data not shown).

4. Discussion

While pan-serotypic rRT-PCR assays for detection of FMDV have been extensively evaluated for routine use (Callahan et al., 2002; Shaw et al., 2004; Moniwa et al., 2007), several challenges face the design of serotype-specific primer/probe sets for rRT-PCR. *In silico* analyses can be used to try to identify conserved sequences for primer/probe binding sites that allow the specific recognition of all viruses within a particular serotype, but do not cross-react with viruses of other serotypes. This process is challenging due to the high variability of the FMDV genome and lack of sequences that are conserved within, but restricted to, a particular serotype. For these reasons, efforts here have concentrated on detecting viruses of particular serotypes from within distinct geographical regions or lineages rather than across all strains globally within a serotype. This current study utilised VP1 coding sequences to design and establish serotype-specific rRT-PCR assays; VP1 is one of the capsid proteins which displays epitope(s) on the virus surface to the host's immune system. VP1 is widely accepted as being the most antigenically significant of the capsid proteins and its sequence can be used to identify the serotype of the strain. The VP1 coding sequence has been the most commonly sequenced and hence it is now the most informative region of the FMDV genome for determining the relationship between different strains for epidemiological analyses.

Initial screening of clinical samples in three different laboratories using singleplex assay formats showed that the serotype-specific primer/probes sets appropriately detected the FMDV genome of serotype O PanAsia-2, serotype A Iran-05 and Asia-1 strains of subgroups 1, 2 and 6 as intended (Table 3 and supplementary data—Annex A). Furthermore, the assays discriminated between viruses belonging to these lineages from viruses of other lineages or subgroups within serotypes O, A and Asia 1, e.g. from other regions of the world. No cross-reactivity was demonstrated in the assays with heterotypic serotypes of FMDV. Equally impressive assay specificity was demonstrated when the primer/probe sets were used in a multiplex assay format (Table 3, supplementary data—Annex B and Fig. 1). Five serotype Asia-1 viruses belonging to the Sindh-08 lineage were not detected with the multiplex format. Sequencing of two of these isolates showed that this was due to the positioning of two mismatches in the probe-binding region (Table 4). Using either assay format, the specific primer/probe sets were also able to identify the serotype of samples designated FMDV-GD, thereby providing important additional epidemiological

information for these samples in the absence of any serotyping data provided by the combined use of Ag-ELISA and VI.

The analytical sensitivity of the specific primer/probe sets as determined by testing serial dilutions of RNA and plasmid standards demonstrated the wide dynamic range of the assays. As with any other RNA virus with a rapidly evolving genome, continuous monitoring of the primer and probe sequences of the serotype-specific sets against the corresponding sequences of newly circulating and emerging viruses will be essential to ensure that the assays maintain fitness for purpose. However, the results from this study clearly demonstrate the potential of rRT-PCR for routine and robust detection and discrimination of FMDV isolates belonging to geographically distinct lineages and sub-groups of serotypes O, A and Asia-1 currently circulating in the Middle East.

Previously, a lineage-specific rRT-PCR has been developed for use in North African countries to specifically detect SAT 2 viruses that emerged from sub-Saharan Africa during 2012 and to differentiate these viruses from endemic FMD virus strains that might be present in these countries (Ahmed et al., 2012). These findings support the use of this approach to develop assays to detect other topotypes of FMDV serotypes O, A and Asia-1, and also for other serotypes, especially SAT 2 as viruses of this serotype have more sequence variation in the VP1 coding sequence compared with viruses of serotypes O, A and C. This current study is the first to describe the use of multiple lineage-specific rRT-PCRs for the detection and discrimination of FMDV in clinical samples in endemic settings. These assays represent the first step towards the development of a suite of molecular tools (molecular toolbox) for different countries and regions. A similar approach could therefore be used to develop FMDV-serotyping assays for other geographical regions of the world.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jviromet.2014.07.002>.

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